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A STUDY ON THE ROLE OF THE BRAIN IN THE ESTABLISHMENT OF ADAPTATION TO REPEATED IMMOBILIZATION STRESS
PART 1
CHANGES IN BRAIN ACTIVITY AND BODILY FUNCTIONS UNDER REPEATED IMMOBILIZATION STRESS

Masahiro Yanase

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16. Abstract A 6-hour immobilization was applied to rabbits for 7 successive days and the establishment of adaptation was investigated. Decreases in food intake, water intake and urine volume in the early stage of the application were reversed after repeated stresses. Although the first immobilization effected decreases in rectal temperature, glucose tolerance and oxidation of C^{14}-u-glucose and C^{14}-labelled short chain fatty acids in liver slices and changed the response of the heart rate to mecholyl, the seventh immobilization had little effect on these. Therefore, adaptation to the immobilization can be considered to appear after repeated exposures to stress. The threshold of stimulation in the midbrain reticular formation inducing neocortical EEG arousal decreased under the immobilization stress, and that in the dorsomedial nucleus of the thalamus increased. Changes in multiple unit activity in the ventromedial nucleus, anterior hypothalamic area, preoptic area and hippocampus under the first immobilization stress diminished with repeated stresses, while those in arcuate nucleus, posterior hypothalamic area and midbrain reticular formation were observed under both the first and seventh stresses.

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I. Introduction

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Ever since it was learned that when an organism is exposed to stress, ACTH is discharged from the pituitary gland and there occurs a series of reactions forming an important process for the establishment of adaptation to stress, many studies have been made concerning the regulation mechanism of secretion of ACTH. Fortier et al. [3] pointed out that when the pituitary glands of mice were removed from the brain and transplanted in the anterior chamber of the eye, even though the transplanted pituitary still had the ability of secreting ACTH, there was no longer observed any increase in the secretion of ACTH as a result of immobilization stress. In view of this fact, they established that the brain plays an important role in the discharging of ACTH in emotional stress such as immobilization stress.

Concerning the role of the brain in regulating the secretion of ACTH, there are numerous reports indicating that the hypothalamus has an intimate relationship [14, 15, 21]. A pioneering report in this respect was that of de Groot and Harris [7]. Today, research has developed into studies concerning the existence and the physiological significance of an ACTH-releasing factor which functionally connects the hypothalamus and the pituitary [35, 45]. The results of electric stimulus tests [10, 17, 49] and destructive tests [31, 38] have shown that there are other positions in the brain besides the hypothalamus which play a role in accelerating

*Numbers in the margin indicate pagination in the foreign text.

the secretion of ACTH. For instance, the pericerebral positions have such a function. In addition, results of electric stimulus tests [11, 24, 51] and destructive tests [9, 19] have been reported which indicate that the midbrain reticular formation also has functions of accelerating the secretion of ACTH, and it has become clear that a broad range of brain positions plays a role in the regulation of ACTH secretion. On the other hand, Egdahl [9] established that dogs whose cerebral cortex and midbrain had been severed had a corticoid concentration in the blood much /110
higher than normal animals. On the basis of this, he reported that the higher brain operates to inhibit the secretion of ACTH with respect to the lower brain. Attention has come to be focused on the role of the hippocampus in the ACTH-secretion-inhibiting mechanism. For example, Porter et al. [13] reported that eosinopenia ceased to be observed as a stress reaction when the hippocampus was given electrical stimuli. Then Mason et al. [39] reported that stimulation of the hippocampus inhibited the increase in the 17-hydroxycorticoid concentration in the blood when stimuli were applied to the hypophyseal stalk. Endröczi et al. [12] also reported that after stimulation of the hippocampus, eosinopenia ceased to be observed as a result of pain, formalin, epinephrine, histamine, and other types of stress. At the same time, it was reported [32] that when various stresses were applied for prolonged periods, the pronounced increase in ACTH discharge which was seen at the beginning ceased after the stresses were applied for a long time, and attention came to be focused on this sort of ACTH release inhibition phenomenon in connection with the inhibition mechanism of ACTH secretion in the brain.

Kawakami et al. [25, 26] reported that when repeated immobilization stresses were applied to rabbits for 7 days, 6 hours a day, there were pronounced increases after the first immobilization in the corticosterone concentration in the plasma and in the absorption of C^{14}-1-acetic acid in the adrenal homogenate into the

corticosterone and the 17-hydroxycorticosterone (17-OHCS). However, after the seventh immobilization, there was, on the contrary, a decrease. At this time it was observed that the susceptibility of the adrenal cortex to ACTH is increased remarkably as a result of repeated stresses. They also report that electrical stimulation of the hippocampus accelerates the secretion of ACTH when the animal is in a state of rest, but that, on the contrary, it inhibits the secretion of ACTH when the animal is under stress. These facts point strongly to the fact that the brain mechanisms play a positive role in the process by which the organism establishes adaptation to repeated stress loads. However, it is believed that the adapation of the organism to stress is established by a broad range of regulatory functions of the organism operating in an adequate manner. At the present time, there are few reports elucidating the functions of the organism during repeated stress from this point of view.

In this study, an attempt was made to study the adaptation of the organism to repeated immobilization stress with respect to the fundamental mechanisms of the organism such as ingestion of food and water, urination, and maintenance of body temperature, and also with reference to the autonomic nerve functions and the sugar and lipid metabolism. An attempt was also made to elucidate the brain activity during the process of applying repeated stresses by the electrophysiological method.

II. Materials and Methods

One hundred and twelve white, grown female rabbits of the New Zealand strain (body weight 2.8-3.5 kg) were used as the test animals. The animals were given water and artificial feed (RC-5, Oriental Kobo Kogyo K.K.) individually in cages in a room with a temperature regulated at 24 ± 0.5°C under artifical lighting for 14 hours a day (lights were kept on from 5:00 AM until 7:00 PM).

As the stress, the rabbits were laid on their backs and their limbs were tied down to immobilize them. This immobilization stress was applied repeatedly for 7 days, 6 hours every day. The stress loads were applied during the hours from noon until 6:00 PM, that is, the time band when the biosynthesis of corticosterone and 17-OHCS in the adrenal gland has a constant value. This time band was selected in view of the diurnal fluctuations in the secretion of ACTH [27].

Ten rabbits were raised individually in cages for collecting urine specimens. For a period of 7 days before the application of the stress, they were given no food or drink during the time band from noon to 6:00 PM. The daily amounts of food and water ingested and the amounts of urine were measured at noon in order to calculate the mean daily amount per rabbit. The values were compared with the daily mean amounts of food and water ingested and the amounts of urine per rabbit measured similarly during the process of repeated immobilization stress.

In six rabbits, a rectal thermometer was used to measure the rectal temperature at 30-minute or 1-hour intervals during the 3 hours before the application of stress, during the application of stress, and during the 2 hours after the stress.

In order to study the effects of the repeated immobilization on the autonomic nervous functions, ten rabbits were given intramuscular injections 0.1 ml of 0.5% methacholine chloride (manufactured by Daiichi Kagaku) 3 days before the application of the repeated stress, 5 hours after the commencement of the first immobilization stress load, and at the same time as the seventh load was applied. The electrocardiograms were recorded, and the changes in the heart rate were studied.

Glucose tolerance tests were performed by intravenously injecting 2 ml of 50% glucose 3 days before the commencement of the repeated immobilization loads in a state of rest without restraints, on the 4th hour after the application of the first stress load, and on the 4th hour after the application of the seventh load. Blood samples were obtained with the passage of time by puncturing the veins in the ears. The blood samples were collected in glass capillary tubes treated with sodium fluoride, and the fluctuations in the blood sugar values were measured by the orthotoluidine boric acid method [46] using the ultratrace system. These tests were made for six rabbits. /111

Forty rabbits were divided into the following four groups of ten rabbits each: a group to which no stress was applied; a group to which the first immobilization stress was applied; a group to which six immobilization stress loads were applied and the seventh load was not applied; and a group to which the seventh load was applied. The rabbits were killed by decapitation, their livers were extracted; and examinations were made concerning the utilization of the glucose in the liver slices and the formation of glucose and lipids from the low-grade fatty acids. In these examinations, various C^{14}-labeling substrates were used, such as C^{14}-u-dextrose, C^{14}-1-acetic acid, C^{14}-2-acetic acid, C^{14}-1-propionic acid, C^{14}-1-butyric acid, C^{14}-2-butyric acid, and C^{14}-3-butyric acid. Liver slices of 2.0 g were subjected to shake culture in 10 ml of Krebs-Ringer bicarbonate buffer solution of pH 7.2 containing 100 µmol (1 µc) of C^{14}-labeling substrate at 38°C for 3 hours in a vapor phase of O_2:CO_2 (95:5). The $C^{14}O_2$ was absorbed into Hyamine, and the Jones method [23] was applied to the glucose to measure the C^{14} activity in the various lipid fractions of cholesterol ester, triglyceride, free cholesterol, and phospholipid. The measurements of the C^{14} activity and of the tissue-N were made by the method of Seto et al. [50]. The consumption of glucose in the liver slices was expressed in terms

of the radioactivity found by subtracting the radioactivity of the residual C^{14} substrate from the radioactivity of the C^{14}-u-glucose which was added.

Forty rabbits were used to examine the brain activity in repeated immobilization stress. Electrophysiological methods were used to measure the EEG arousal threshold of stimulation of the frontal cortex as a result of stimulation of the ventromedial region of the midbrain reticular formation and the dorsomedial region of the thalamus and to record the MUA at various positions in the brain. The electrodes used for the various experiments were implanted in the following manner. The animals were anesthetized by means of pentobarbitol sodium and fastened in a brain-positioning device. The electrodes were then implanted permanently and fixedly at the various positions in the brain in accordance with the rabbit brain atlas compiled by Sawyer et al. [47]. After the experiments were finished, the electrode positions were confirmed histologically. Concentric type bipolar electrodes made of stainless steel were used as the stimulating electrodes, and silver ball electrodes were used for recording the electrocardiograms. The electrodes for recording the MUA were made by electrolytically polishing insect needles (N_oO manufactured by Shiga Konchu) until their tips were about 20 μm, and then coating with Epoxyrite (manufactured by Dai Nippon Yushi). The experiments were started after a period of 2 or 3 weeks after the operations, when the animals had already recovered from the operative trauma. As for the EEG arousal threshold of stimulation, square-wave stimuli of 100 Hz and a stimulus width of 0.1 msec were applied for 5 seconds by means of an electric stimulus device (model MSE-3, manufactured by Nippon Koden), and the stimulus voltage threshold value was measured, using the changes in the brain waves as the index. As for the MUA record, the MUA was put through a solid-state preamplifier, and the wave forms were observed by means of a cathode-tube oscilloscope (Model 80-207, manufactured by San'ei Sokki) by the methods of Sawyer et al.

[48] and Kawakami et al. [28]. In addition, they were integrated by means of an RC integrator, and the DC components were drawn out on a polygraph (manufactured by San'ei Sokki). The electrocorticograms were also recorded at the same time on the polygraph and were compared with the MUA.

III. Results

A. Effects of Repeated Immobilization Stress on Amounts of Food and Water Ingested and on Urine Volume

In the first mobilization stress, the animals moved their bodies hardly at all immediately after the stress was applied, and their pupils were greatly contracted. The miosis recovered as the stress continued to be applied, and after about 2 hours had gone by after the stress began to be applied, the pupils were medium-large. Beginning about 10 minutes after the load began to be applied, the animals were seen to make intermittently intense stretching and contracting movements lasting about 10 seconds each. During the first load, borborygmus and defecation were frequently observed, but this is believed to have been the result of accelerated intestinal movement. In most cases, the heart rate became rather frequent immediately after the application of the load, but in many cases it gradually decreased as the load continued to be applied. As the immobilization loads were repeated, the miosis, borborygmus, defecation, and intermittently occurring intense stretching and contracting movements which were observed conspicuously during the first immobilization load ceased to be very conspicuous, and during the seventh load these phenomena could hardly be observed at all. However, miosis was observed to a considerable degree immediately after the seventh load. After they were released from immobilization after the first load, most of the animals would crouch without moving in a corner of their cages for about 1 hour. However, after the seventh load, /112 immediately after being released, they would move about their cages just as in normal conditions.

TABLE 1. BODY WEIGHT, FOOD AND WATER INTAKE AND URINE VOLUME EACH DAY IN THE COURSE OF THE REPEATED IMMOBILIZATION STRESS. EACH VALUE IS THE MEAN FOR TEN RABBITS, AND THE CONTROL IS THE MEAN FOR 7 DAYS BEFORE THE STRESSFUL DAYS IN THE SAME GROUP OF RABBITS.

	Control	Repeated immobilization stress						
		1st day	2nd day	3rd day	4th day	5th day	6th day	7th day
Body weight kg	3.25±0.16	2.90	2.86	2.88	3.05	3.20	3.09	3.13
Food intake g/day	106±9	50	46	101	102	113	119	101
Water intake ml/day	340±11	223	228	313	364	358	361	339
Urine volume ml/day	182±35	129	126	142	172	150	176	169

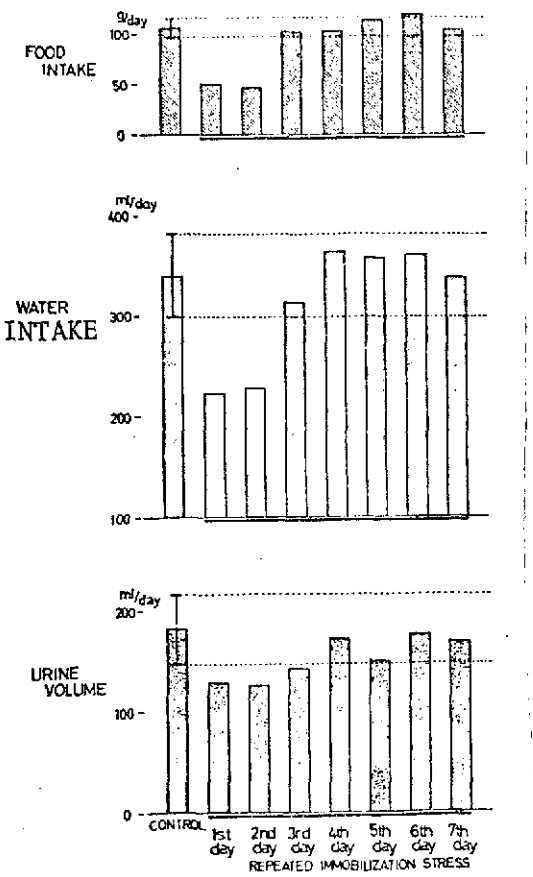

Fig. 1. Changes in body weight, food and water intake and urine volume in the course of the repeated immobilization stress. I indicates the standard deviation for 7 days before the stressful days.

The effects of repeated immobilization stress on the body weight are shown in Table 1. The mean body weight of a group of ten animals for a 7-day period before the repeated loads was 3.25 kg with a standard deviation of ±0.16 kg. No significant difference could be observed between this and the mean body weight during the repeated loads, although a tendency toward slight decrease in body weight could be observed until the day when the third load was applied.

The changes in the mean amounts of daily food and water intake and in the urine volume of this group of animals are shown in Table 1 and Fig. 1. During the 7 days before the application

of the repeated immobilization stress, the mean amount of daily food intake was 106 g with a standard deviation of ±9 g. There were significant decreases on the 1st and 2nd days of load application, with mean amounts of 50 g and 40 g per animal, respectively. After this, there was gradual recovery, and on the 5th and 6th days, there was observed a tendency for the values to be larger than those in the control group. As for the water intake, during the 7 days before the application of the repeated immobilization stress, the mean amount of daily water intake was 340 ml with a standard deviation of ±41 ml. The mean water intake on the first 2 days of application of loads was 223 ml and 228 ml. This marked a significant decrease in comparison with the control group. Subsequently, the significant difference disappeared. The mean daily urine volume per animal in the control group was 182 ml with a standard deviation of ±35 ml. On the other hand, there were significant decreases during the 1st, 2nd, and 3rd days of loading, the values on those days being 129 ml, 125 ml, and 141 ml. Subsequently, there was gradual recovery, and after the 4th day, there were no differences from the control group (Table 1, Fig. 1).

B. Effects of Repeated Immobilization Stress on the Rectal Temperature

The rectal temperature measured every hour beginning 3 hours before the application of the first immobilization load was constant for each individual and was in the range of 39.0-39.6°C. After the first load was applied, the rectal temperature gradually declined in all cases, reaching the minimum value after 1-2 hours of loading. Subsequently, this low value was maintained, but the value before the application of the load was recovered within 1-2 hours after release from the load. In some individuals, the decline in the rectal temperature was pronounced, the difference /113 from the value before loading amounting to 0.6-2.5°C. After the loads had been repeated, the pronounced decline in the rectal temperature seen during the first load disappeared almost completely.

That is, during the 3 hours before the application of the seventh immobilization load the rectal temperature was constantly 39.2-39.6°C. During the seventh load, in five out of six cases there was a decline of only 0.1-0.3°C in comparison with the value before the load, and in one case there was a decline of 1.3°C. However, in this same case the decline in the rectal temperature had reached 2.5°C during the first load, and the phenomenon of a reduction of the degree of rectal temperature decline during loading as the loads were repeated was the same as in the other cases. After release from the seventh load, the rectal temperature returned to the value before loading in the same way as in the first load (Fig. 2).

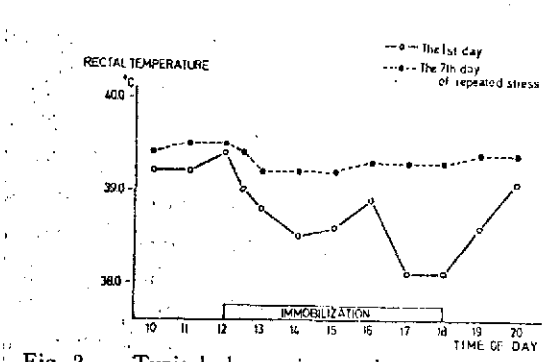

Fig. 2. Typical change in rectal temperature under the immobilization stress in the course of the repeated exposure.

C. Effects of Repeated Immobilization Stress on the Response of the Heart Rate to Methacholine Chloride

When 0.1 ml of methacholine chloride was administered to rabbits in a state of rest before the application of the repeated stress, in five cases there was a decrease in the heart rate during the first 5-10 minutes after administration, or there was no change for a certain time. After this period, there was an increase, returning within 1 hour after administration to the heart rate which applied before administration. In three cases, the heart rate began to increase 1-3 minutes after administration and returned to the original value within 1 hour. In two cases, the heart rate decreased after administration of methacholine chloride and returned to the original value within 1 hour, but no pronounced increases above the heart rate before administration were observed.

These changes of the heart rate with respect to methacholine chloride administered in a state of rest differed pronouncedly from the changes in the heart rate after methacholine had been administered during the first immobilization load. In those cases which had displayed an increased heart rate within 5-10 minutes after the administration of methacholine in a state of rest, there was an increase in the heart rate immediately after administration during the first load, and the degree of increase was also pronounced. In those cases which had displayed pronounced increases in the heart rate immediately after methacholine had been administered in a state of rest, almost no increases in the heart rate were observed when methacholine was administered during the first immobilization load. In those cases which had displayed decreases in the heart rate when methacholine was administered in a state of rest, almost no increases in the heart rate were observed when methacholine was administered during the first immobilization load. In those cases which had displayed decreases in the heart rate when methacholine was administered in a state of rest, there were increases in the heart rate when methacholine was administered during the first load. After repeated applications of stress, the changes in the heart rate caused by administration of methacholine chloride resembled those occurring in a state of rest, and the changes in the heart rate after administration of methacholine chloride during the seventh immobilization load in all cases tended to resemble the reactions displayed in a state of rest before the repeated application of loads (Fig. 3).

D. Effects of Repeated Immobilization Stress Loads on the Glucose Tolerance Tests

The mean blood sugar value before the application of glucose loads was 119 mg/dl with a standard deviation of ±15 mg/dl at the state of rest before repeated immobilization loads. Ten minutes

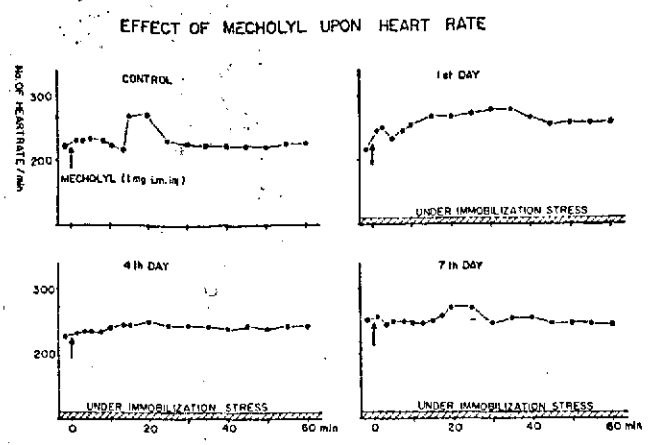

Fig. 3. Typical changes in the response of the heart rate to methacholine chloride (mecholyl) in the course of repeated immobilization. The control is the response in a state of rest on a non-stressful day.

after the application of glucose loads, the blood sugar increased to about 70 mg/dl. Then, after 30 minutes of loading, it returned to a value of 126 ± 13 mg/dl, with no significant difference from the control group, and constant /114 values continued to be displayed after that. During the application of the first immobilization stress load, there was a blood sugar value of 117 ± 18 mg/dl before administration, but there was a conspicuous increase in the blood sugar after 10 minutes of sugar loading. Even after 30 minutes of sugar loading, the value was 142 ± 17 mg/dl, a blood sugar value significantly higher than that in the control group. Even after 120 minutes of sugar loading, a significantly higher value of 142 ± 17 mg/dl was maintained. During the seventh immobilization stress load, there was an increase in the blood sugar value after 10 minutes of sugar loading, while the control group had a blood sugar value of 112 ± 9 mg/dl. Then, after 30 minutes of loading, the blood sugar value increased to 129 ± 18 mg/dl, and it was no longer possible to perceive any significant difference from the control group (Table 2, Fig. 4).

Table 2. Glucose tolerance test under immobilization stress. Mean glucose level in plasma (mg/100ml) ± standard deviation of six experiments is given

	Blood glucose level after the injection of 2ml of 50% glucose					
	Control	10	30	60	90	120 min
In state of rest	119±15	186± 9	126±13	123± 1	121±10	120± 8
Under 1st stress	117±18	195±17	175±16	146±20	140±20	142±17
Under 7th stress	112± 9	186±18	129±18	119± 9	115± 9	112±10

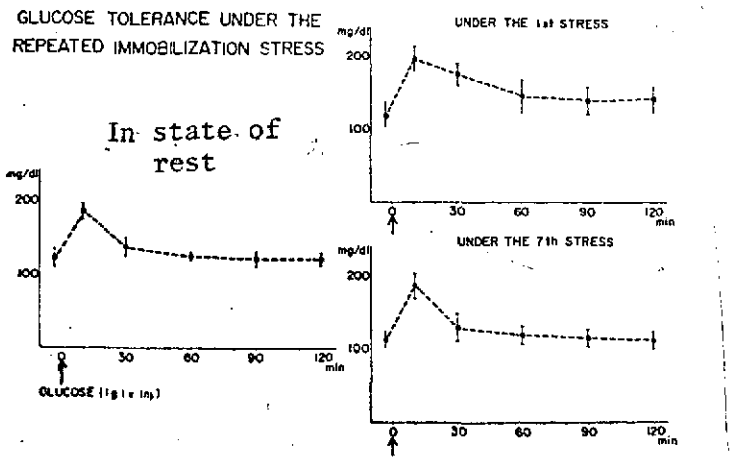

Fig. 4. Graphic representation of the results of the glucose tolerance test in the course of repeated immobilization stress. I indicates mean blood glucose level and standard deviation for six rabbits.

E. Effects of Repeated Immobilization Stress on the Metabolism of Glucose and Low-Grade Fatty Acids in Liver Slices

As is shown in Table 3, in the group given the first immobilization load the consumption of C^{14}-u-glucose was less than in the group given no immobilization load. There was also a pronounced decrease in the CO_2 formation and in the absorption into the various lipid fractions. After repeated application of loads, in the group given the seventh load there was no significant difference in the consumption of C^{14}-u-glucose in the liver slices, in the CO_2 formation, or in the absorption into the various lipid fractions in comparison with the group in the state of rest on the 7th day, and the pronounced changes resulting from the first load could no longer be seen in the seventh load. The metabolism of the C^{14}-u-glucose in the liver slices in the state of rest was the same on the 1st day and on the 7th day.

Table 3. ^{14}C-u-glucose consumption and its incorporation into CO_2 and lipids in liver slices under the immobilization stress. Counts±standard deviations (/mc/mg liver tissue protein-N) of ten experiments are shown

Repeated stress	Immobilization (No of experiments)	^{14}C-u-glucose consumption	^{14}C-u-glucose incorporation in liver slices into CO_2	A	B	C	D
1st day	−(10)	4980±52	2558±22	28± 1	31± 1	27± 1	79± 1
	+(10)	4570±30	2104±29	18± 1	30± 1	18± 1	60± 1
7th day	−(10)	4896±28	2472±17	30± 1	31± 1	30± 1	78± 2
	+(10)	5005±32	2468±15	30± 1	31± 1	29± 1	80± 1

A : Fraction of cholesterol ester. B : Fraction of triglyceride. C : Fraction of free cholesterol. D : Fraction of phospholipid.

Table 4. Incorporation of ^{14}C-labelled short-chain fatty acids into glucose, CO_2 and lipids in liver slices under the immobilization stress

Counts±standard deviations (μmc/mg liver tissue protein-N) of ten experiments are shown

Repeated stress	^{14}C-labelled fatty acid incorporation into	Immobilization	^{14}C-labelled short-chain fatty acid						
			acetate		propionate		butylate		
			^{14}C-1-	^{14}C-2-	^{14}C-1-	^{14}C-2-	^{14}C-1-	^{14}C-2-	^{14}C-3-
1st day	glucose	−	196± 4	603± 3	825± 2	2714±20	210± 4	819±5	234± 2
		+	254± 3	903± 5	1557±21	5760±29	202± 3	842±2	314± 2
	CO_2	−	2004±11	839± 7	4193±21	648± 2	3112±47	225±4	1947±22
		+	1477±27	717±10	3429±30	506± 5	2896±18	342±2	2899±11
	cholesterol ester	−	50± 1	110± 1	19± 1	25± 1	28± 2	90±1	31± 1
		+	29± 1	41± 1	13± 1	18± 1	31± 1	87±2	44± 1
	triglyceride	−	20± 1	51± 1	11± 1	12± 1	31± 1	79±1	30± 1
		+	13± 1	19± 1	9± 1	11± 1	31± 1	96±2	45± 1
	free cholesterol	−	59± 1	121± 1	15± 1	19± 1	32± 3	79±2	29± 1
		+	30± 1	50± 1	11± 1	10± 1	31± 1	86±1	44± 2
	phospholipid	−	101± 1	251± 2	42± 1	64± 1	82± 1	181±2	80± 1
		+	59± 1	120± 2	29± 1	37± 2	81± 2	202±2	110± 2
7th day	glucose	−	200± 3	590± 4	835± 3	3288±23	204± 3	825±2	262± 3
		+	200± 2	611±12	837± 3	3398±20	206± 3	838±2	299± 3
	CO_2	−	1991±12	826± 4	4148±33	607± 3	2993±12	243±2	2397±21
		+	2002±10	821± 5	4176±36	600± 3	2997±11	253±2	2460±20
	cholesterol ester	−	51± 1	111± 3	17± 1	22± 1	31± 1	84±1	36± 1
		+	51± 1	111± 3	16± 1	15± 1	31± 1	88±1	42± 1
	triglyceride	−	21± 1	50± 1	11± 1	13± 1	30± 1	90±2	34± 2
		+	31± 1	55± 2	51± 1	14± 1	31± 1	92±2	39± 1
	free cholesterol	−	53± 1	120± 3	15± 1	19± 1	30± 1	83±1	33± 1
		+	55± 2	120± 1	14± 1	19± 1	31± 1	84±1	40± 2
	phospholipid	−	99± 2	244± 4	37± 1	60± 1	80± 1	180±1	91± 1
		+	104± 2	244± 3	40± 1	50± 1	82± 1	190±1	102± 3

Data concerning the metabolism of low-grade fatty acids are shown in Table 4. In the group given the first immobilization load, there was an increase in the absorption of the C^{14}-1-acetic acid and the C^{14}-2- acetic acid into the glucose in comparison with the group which was not given immobilization loads; there was also a decrease in the CO_2 formation and in the absorption into the various lipid fractions. Changes in which there were increases in the amounts absorbed into the glucose, decreases in the CO_2 formation, and decreased absorption into the various lipid fractions were also observed in the case of C^{14}-1-propionic acid. However, in the group given immobilization loads, there was a certain tendency toward decrease in the absorption of the C^{14}-2-propionic acid into the glucose, while there was an increase in the CO_2 formation. The /115 metabolism of C^{14}-1-butyric acid was not affected by the first immobilization, but the absorption of C^{14}-2-butyric acid and C^{14}-3-butyric acid into the glucose increased in the immobilized groups,

and with C^{14}-3-butyric acid there were increases in the absorption into the various lipid fractions and in the CO_2 formation. The pronounced effects of the first immobilization load on the metabolism of the low-grade fatty acids had almost ceased to be observable in the seventh immobilization load. That is, the absorption of the low-grade fatty acids into the glucose, the CO_2 formation, and the absorption into the various lipid fractions in the state of rest on the 7th day of the immobilization load were almost exactly the same as those in the state of rest on the 1st day, but even after the application of the seventh immobilization load, it was not possible to observe the pronounced changes which occurred after the application of the first immobilization load.

F. Effects of Repeated Immobilization Stress Loads on the EEG Arousal Threshold of Stimulation

As is shown in Fig. 5, the cortical EEG arousal threshold by /116
stimulation of the medial area of the midbrain reticular formation was 0.8-1.2 V before the application of the immobilization load on the first day. As a result of the first load, the threshold value decreased to 0.4-1.0 V, and this value was retained during the application of the load. Within 1-2 hours after release from the load, the value returned to the value before application of the load. This decline of the threshold value was observed even when loads were repeated. That is, in the state of rest on the 7th day, the EEG arousal threshold of stimulation resulting from stimulation of the midbrain reticular formation was almost the same as that on the 1st day, and when the seventh load was applied there was still observed a decrease in the threshold value in the same manner as when the first load was applied. On the other hand, the cortical EEG arousal threshold of stimulation when the dorsomedial nucleus of the thalamus was stimulated was 0.5-0.7 V in the state of rest on the 1st day. However, when the first load was applied, an increase in the threshold value was observed, a high value of 0.3-0.5 V was maintained during the application of the load, and

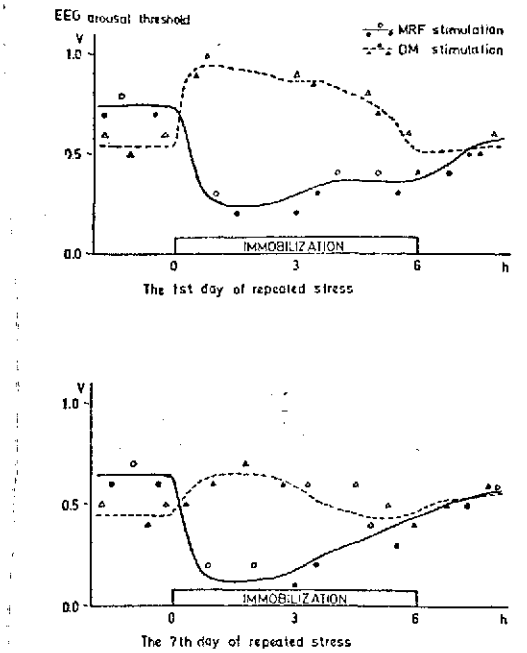

Fig. 5. Changes in the EEG arousal threshold of stimulation in the ventromedial region of midbrain reticular formation (MRF) and in the dorsomedial nucleus of thalamus (DM) under the immobilization stress.

the value returned to the original value within 2 hours after the removal of the immobilization load. After repeated immobilization loads, in the state of rest on the 7th day, the EEG arousal threshold of stimulation was exactly the same as that in the state of rest on the 1st day. However, the increase of the threshold value on account of application of the seventh load was limited to 0.1-0.3 V, and after 3 hours after the commencement of loading, the value became approximately the same as that before the application of the immobilization load.

G. Effects of Repeated Immobilization Stress Loads on the MUA in Various Positions in the Brain /117

The MUA was recorded during the process of applying the first and the seventh immobilization loads, and comparisons were made of the MUA at the moments when the cortical EEG, which was also recorded simultaneously, indicated the slow-wave sleep phase. When the MUA at each of the brain positions were compared for the periods when the cortical EEG indicated the slow-wave sleep phase, it was found that a more or less constant level was maintained when the animals were in an unrestrained state. The positions where the MUA was recorded are shown in Fig. 6.

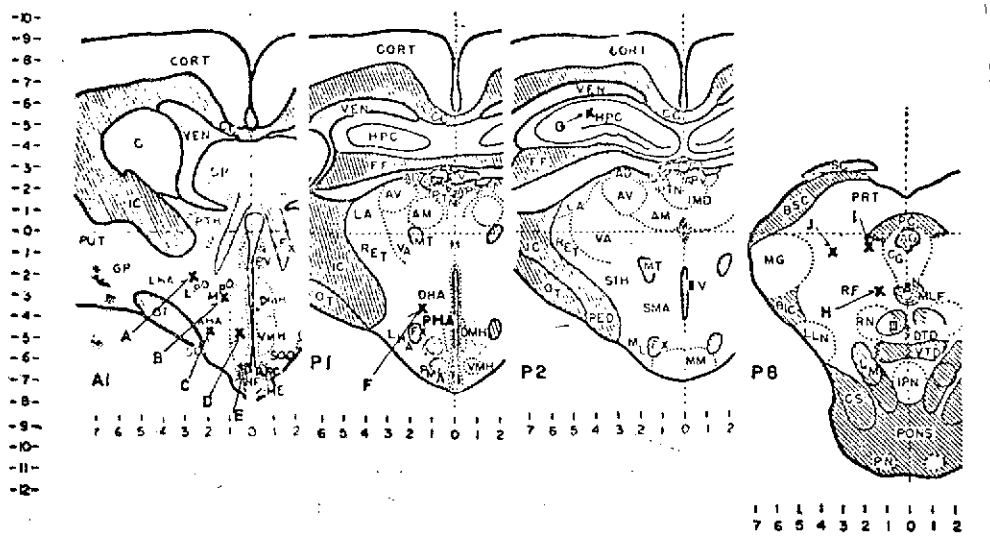

Fig. 6. The location of the tips of electrodes. A : Lateral preoptic area (LPO). B : Medial preoptic area (MPO). C : Anterior hypothalamic area (AHA). D : Ventromedial nucleus of hypothalamus (VMH). E : Arcuate nucleus of hypothalamus (ARC). F : Posterior hypothalamic area (PHA). G : Hippocampus (HPC). H : Ventromedial region of midbnain reticular formation (Ven. Med. mRF). I : Dorsomedial region of midbrain reticular formation (Dor. Med. mRF). J : Dorsolateral region of midbrain reticular formation (Dor. Lat. mRF).

a. Hypothalamus

The MUA of the arcuate nucleus of the hypothalamus (ARC) began to decline around 20 minutes after the application of the first immobilization load. It reached the minimum level within 2 hours after application. Subsequently, it displayed a certain tendency to increase, but throughout the entire 6 hours of the immobilization load, it maintained a level lower than before the load was applied. For longer than 1 hour after release from the load, no slow-wave sleep phase appeared in the cortical EEG. The slow-wave sleep phase appeared only afterward. The MUA of the arcuate nucleus of the hypothalamus returned to the level before the application of the load within 2 hours after release from the immobilization load. After the application of the seventh immobilization load, the MUA of the arcuate nucleus of the hypothalamus began to decline after about 20 minutes, just as when the first load was applied, and the minimum value was maintained until 3 hours after application of the load. Then, it gradually approached the level before application of

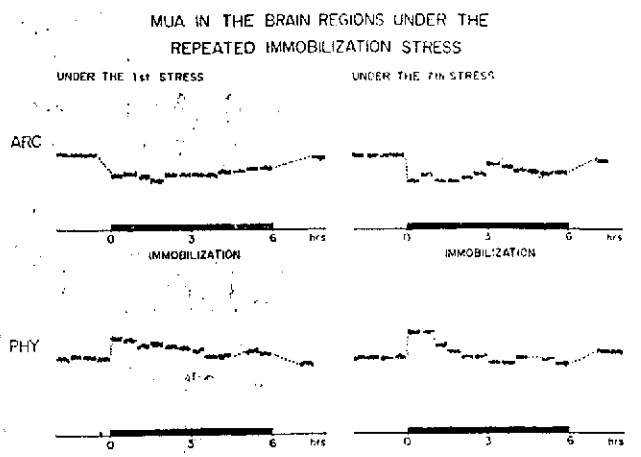

Fig. 7. Multiple unit activity in the arcuate nucleus (ARC) and the posterior hypothalamic area (PHY) under the repeated immobilization stress.

the load. However, the low level was maintained during the entire application of the load. After 1 hour after release from the load, the value returned to the level before application of the load (Fig. 7).

The MUA of the posterior hypothalamic area (PHY) maintained its highest level from 20 minutes to 1 hour after the application of the first immobilization load. Subsequently, the value gradually declined and came close to the level before application of the load. However, during the application of the immobilization load, the level was higher than it had been before the application of the load. Within 1-2 hours after release from the load, it had returned to the level before application of the load. As for the changes in the MUA of the posterior hypothalamic area resulting from the application of the seventh immobilization load, a rise was observed for a period of 1 hour, beginning immediately after the application of the load, just as when the first load was applied. However, the values subsequently declined gradually and approached the level before the application of the load. After loading for 3 hours, the values came to have almost the same level as that before the application of the load. However, after release from the load, there was a slight increase, but after 3 hours after release, the value returned to the level before application of the load (Fig. 7).

The MUA of the ventromedial nucleus of the hypothalamus (VMH) /118 increased immediately after the application of the first immobilization load. Within 1 hour, it decreased slightly, and a level higher

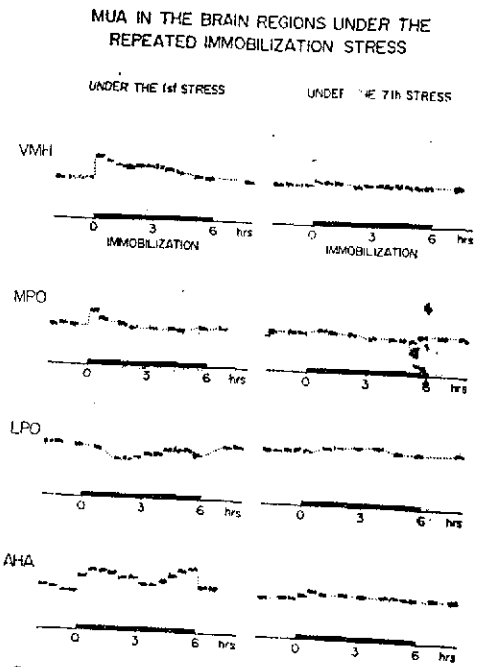

Fig. 8. Multiple unit activity in the ventromedial nucleus of hypothalamus (VMH), medial preoptic area (MPO), lateral preoptic area (LPO) and anterior hypothalamic area (AHA) under the repeated immobilization stress.

than that before the application of the load was maintained until about 5 hours after the application of the load. However, after that, it declined gradually and returned to the level before the application of the load. After release from the load, the level before the application of the load was maintained unchanged. In the seventh immobilization load, the changes which were seen as a result of the application of the first immobilization load could not be observed, and the MUA of the ventromedial nucleus of the hypothalamus remained more or less constant throughout the entire process of the seventh load (Fig. 8).

The changes resulting from the first immobilization load in the MUA derived from the anterior hypothalamic area (AHA) were as follows. There was an increase beginning immediately after the application of the load. A high level was maintained, and after about 3 hours after the application of the load there was a slight decrease. Subsequently, the increase was resumed, and a high level was maintained throughout the application of the immobilization load. After release, there was a rapid return to the level before application of the load. These changes were not seen during the process of application of the seventh immobilization load, at which time more or less the same level was maintained (Fig. 8).

The MUA of the medial preoptic area (MPA) increased for approximately 1 hour beginning immediately after the application of the first immobilization load. Subsequently, it declined gradually, and after 2 hours after the application of the load, it returned to the level before the application of the load. This level was maintained and did not change even after release. After the application of repeated immobilization loads, there were no changes in the MUA at this position when the seventh immobilization load was applied, and a constant level was maintained throughout the process of application of the load (Fig. 8).

In the lateral preoptic area (LPO), there was observed a decline of the MUA beginning about 1 hour after the application of the first immobilization load, and this low level was maintained until about 4 hours after the application of the load. However, subsequently it returned to the level before application of the load, and even after release it remained at the level before application of the load. The decline in the MUA at this position which was seen as a result of the application of the first load was not observed in the seventh load, and the MUA maintained a more or less constant level during the process of the seventh immobilization load (Fig. 8).

b. Pericerebral System

The MUA recorded from the dorsal hippocampus (HPC), during the entire application of the first immobilization load, maintained a higher level than that before application of the load. That is, the MUA increased immediately after application of the load, and this high level was maintained from 2 to 3 hours after the application of the load. Then it declined gradually, and after release from the load, it returned to the MUA level before application of the load. After immobilization loads had been repeated, no changes in the MUA of the hippocampus could be observed, and during the

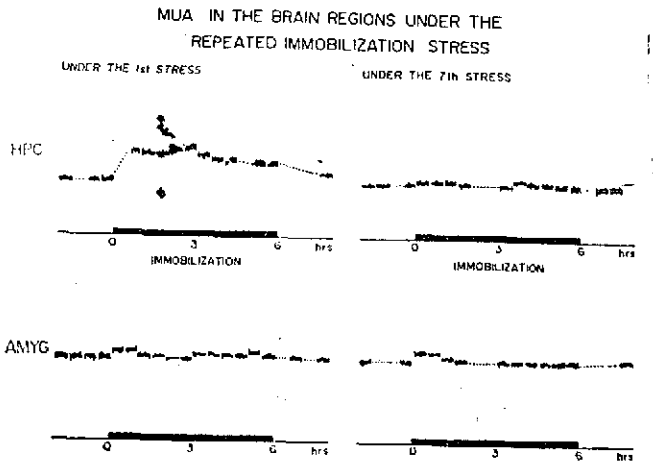

Fig. 9. Multiple unit activity in the hippocampus (HPC) and amygdalar (AMYG) under the repeated immobilization stress

entire process of application of the seventh immobilization load, the MUA at all times was at approximately a constant level.

No changes could be observed in the MUA of the amygdalar (AMYG) either during the first immobilization load or in the seventh immobilization load (Fig. 9).

c. Midbrain Reticular Formation

As for the changes caused by immobilization loads in the MUA of the midbrain reticular formation (mRF), pronounced differences were observed in accordance with the positions from which the records were derived. In the following description, a position 2 mm from the median line was used as the demarcation line between the medial and the lateral areas. The height of the oculomotor nerve nucleus was taken as the ventral side, and the height of the aqueduct of the midbrain was taken as the dorsal side. Thus, the midbrain reticular formation was divided into the ventromedial region (VEN. MED. mRF), the dorsomedial region (DOR. MED. mRF), and the ventrolateral region.

The MUA derived from the ventromedial region of the midbrain reticular formation began to increase gradually beginning immediately after the first immobilization. A pronounced increase was observed, reaching an apex around the 6th hour of application of the load. When the load was released, the values gradually began to return to the level before application of the load, and after 1 to 2 hours after release, they had returned almost entirely to the level before

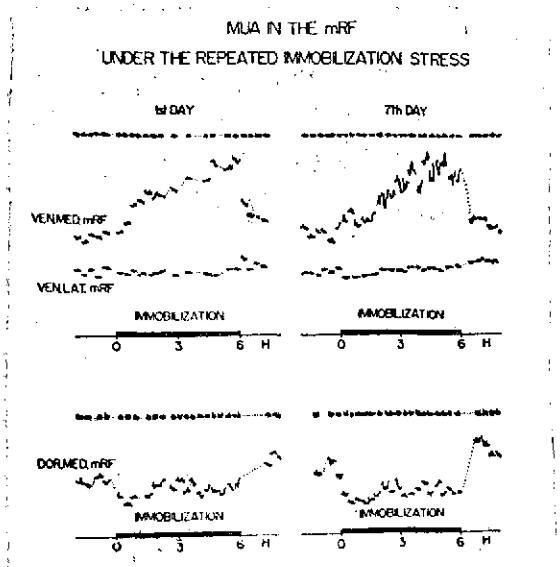

Fig. 10. Multiple unit activity in the ventromedial (VEN. MED. mRF), ventrolateral (VEN. LAT. mRF) and dorsomedial (DOR. MED. mRF) regions of midbrain reticular formation under the repeated immobilization. Simultaneous record of cortical EEG is shown above.

application of the load. On the other hand, the MUA of the dorsomedial region of the /119 midbrain reticular formation began to decrease immediately after the application of the first immobilization load, and at around the 2nd or 3rd hour of application of the load, the level before application of the load was maintained again. However, there was subsequently another decline, and a low level continued until release from the load. After release, there was a sudden increase, and even around 2 hours after release, there was a higher level than the level before the application of the loads. The changes in the MUA of the medial region of the midbrain reticular formation which were seen after the application of the first immobilization load were also observed in the same manner even after the application of repeated loads. That is, after the application of the seventh immobilization load, the MUA of the ventromedial region of the midbrain reticular formation gradually increased immediately after the application of the load, in the same way as when the first load was applied, reaching its apex immediately before release. After release, it returned to the level it had before the application of the load. The MUA of the dorsomedial region of the midbrain reticular formation also declined as a result of the application of the seventh immobilization load. This low level was maintained throughout the application of the load, and the high level to which it rose after release was still indicated even 2 hours after release (Fig. 10).

IV. Considerations

Kawakami et al. [25] reported the trends in the secretion of ACTH under repeated immobilization stress loads in rabbits under the same conditions as in these experiments. That is, when the first immobilization stress load was applied, there was a pronounced increase in the discharge of ACTH, amounting to approximately 60% of that in the state of rest. The absorption of the C^{14}-1-acetic acid in the blood corticosterone and in the adrenal homogenate into the corticosterone and the 17-OHCS was used as the index. In spite of this, when the seventh immobilization load was applied after repeated applications, the absorption of the C^{14}-1-acetic acid in the blood corticosterone and in the adrenal homogenate into the corticosterone and the 17-OHCS came to drop below that in the state of rest. It is believed that this phenomenon occurs for the following reasons. That is, when the identical amounts of ACTH are administered to animals which were not given immobilization loads and to animals which had been given seven immobilization loads, and when one compares the increase in the biosynthesis of corticosterone and 17-OHCS in the blood corticosterone and in the adrenal cortex, there is a pronounced increase in them after the repeated immobilization loads. In view of this fact, it is believed that this is attributable to the fact that the repeated immobilization loads cause the secretion of ACTH from the pituitary gland to cease, in spite of the fact that the hormone-secreting capacity of the adrenal cortex has been heightened by the repeated loads. It is also said that an increase in the discharge of ACTH was observed once again when another type of stress was applied -- that is, when formalin was administered -- during the application of the seventh immobilization load, that is, when increase in the discharge of ACTH as a result of immobilization stress ceased to occur [29]. This fact indicates that, even after repeated immobilization loads, the pituitary gland still retains a sufficient ability to discharge ACTH. Consequently, it

is believed that the fact that the phenomenon of increased discharge of ACTH as a result of immobilization stress ceases to be observed when immobilization loads are repeated occurs because, during the process of repeated application of immobilization loads, a mechanism for inhibiting the discharge of ACTH has been established in the brain, which regulates the discharge of ACTH from the pituitary. On the other hand, if one considers the role of ACTH as an adaptation hormone, it is believed that this tendency in the secretion of ACTH in repeated application of immobilization loads is connected with the process of establishing adaptation to immobilization stress in the organism. It is said that the state at which the organism has established adaptation is the state at which the various functions of the organism are operating in order to maintain homeostasis in the internal environment necessary for survival of the organism with respect to external changes [3].

When the organism is exposed to various stresses, changes /120
occur in the secretion of various humoral factors besides ACTH and the adrenal cortex hormone. For example, changes occur in the secretion of epinephrine, acetylcholine, histamine, serotonin, vasopressin, the growth hormones, and the thyrotropic hormone [5, 33, 41, 53]. It has also been reported that there are neural factors playing a role. For instance, the autonomous nervous system functions as an emergency reaction, and the activity of the central nervous system and peripheral nervous system also plays a role [18, 36, 44]. It is believed that these diverse humoral and neural factors exert various influences on the functions of the organism under stress.

In these experiments, it was clearly established that the fundamental functions of the organism such as the ingestion of food and water, urination, and the body temperature maintenance function, as well as the reactions of the autonomic nervous system, the metabolism of sugar and lipids, and the brain activity are all influenced to a pronounced degree by the application of immobilization stress loads.

Concerning the mechanism in the brain for regulating the ingestion of food, Anand et al. [1] point out that animals fall into starvation when the lateral hypothalamus is destroyed and that polyphagia occurs as a result of destruction of the ventromedial nucleus of the hypothalamus. On this basis, they called the lateral hypothalamus the food ingestion center and the ventromedial nucleus the saturation center. When electric stimuli are given to the food ingestion center, there is an increase in the amount of food ingested [8], and when electric stimuli are given to the saturation center, food ingestion actions are discontinued [42]. In view of this, the importance of the hypothalamus in the regulation of food ingestion is emphasized. On the other hand, Koikegami et al. [34] see that polyphagia occurs as a result of destruction of the amygdalar; they established clearly that the pericerebral system plays a role in the regulation of food ingestion. As for the brain mechanism for regulating the drinking of water, Andersson et al. [2] view the vicinity of the paraventricular nucleus in the dorsal hypothalamus as the center of thirst. Concerning the secretion of vasopressin, which is an important factor in the regulation of the urine volume, Cross et al. [6] and Brooks et al. [4] report that the emission of neurons in the vinicity of the supraoptic nucleus responds well to the various stimuli which influence the secretion of vasopressin, such as changes in the osmotic pressure. The importance of the hypothalamus in the regulation of moisture is obvious. If we take into consideration the fact that changes in the electric activity are observed in the various positions in the hypothalamus and in the hippocampus in the pericerebral system as a result of the application of immobilization stress, it is believed that the changes caused by immobilization loads in the activities of the mechanisms in the brain for regulating the ingestion of food and water are intimately connected with the fact that there are pronounced decreases in the amounts of food and water ingested and in the urine volume during the initial period of repeated immobilization loads.

The rectal temperature decreased by 0.6-2.5°C when the first immobilization load was applied. However, this fact signifies that the balance between the mechanisms of thermogenesis and of thermolysis was made to shift in the direction of thermolysis as a result of the immobilization loads. In the liver, viewed as a thermogenetic organ, the utilization of C^{14}-u-glucose and the formation of CO_2 decreased as a result of the application of the first immobilization load, and as a result of the glucose tolerance tests stagnation of glucose in the blood was seen during the application of the first immobilization load. These facts indicate that the metabolic activity utilizing sugar and the oxidation activity declined as a result of the application of immobilization loads. Furthermore, butyric acid, propionic acid, acetic acid, and other low-grade fatty acids are substances which are absorbed from the intestinal tract as metabolites produced by the bacteria which are constantly present in the cecum and which are regarded as being especially important as nutrients in herbivorous animals such as rabbits [52]. It was found that, when the first immobilization load was applied, there was increased absorption of the various C^{14}-labeled acetic acids and propionic acids into the glucose in the liver slices, that there was reduced absorption into the lipid fractions, and that there was a decrease in CO_2 formation. These facts indicate that the metabolic activity of the oxidation system of these substances in the liver declined. On the other hand, it is said that the reactions of the thermolytic mechanisms for the body temperature are based chiefly on the functioning of the parasympathetic system. The temporal changes in the heart rate under the influence of methacholine chloride are believed to point to the reactivity of the sympathetic nervous system [16], and this indicates that the changes in the heart rate under the influence of methacholine chloride during the application of the first immobilization load differ from those which occur during the state of rest. It also indicates that the autonomic balance during the application of the first immobilization load differs from that during the state of rest. Pronounced contraction of the pupils and acceleration

of the intestinal movement were seen as a result of the application of the first immobilization load. This fact indicates that the parasympathetic nervous system is in a dominant state during the application of the first immobilization load, and it is believed that this is a state at which reactions of the parasympathetic system tend to occur easily as a thermolytic mechanism. Ever since the heat puncture experiments, it has been supposed concerning the mechanism in the brain for regulating the body temperature that an important regulating mechanism for the body temperature is present in the hypothalamus. It was clearly established by Ranson et al. [36] that the cold center is locally present in the anterior hypothalamic area and that the heat center is locally present from the tuber cinereum towards the lateral area of the corpus mammillaria, and it is thought that the correlated action of the endocrine system and the nervous system consisting chiefly of the pericerebral system, the hypothalamus, the pituitary, and the thyroid system plays a vital role in regulating the body temperature [20]. It is believed that the effects of immobilization loads /121 on these brain mechanisms for regulating the body temperature also play a role in the lowering of the body temperature as a result of immobilization loads.

During the process of application of repeated immobilization stress loads for a period of 7 days, the amounts of food and water ingested and the urine volumes declined during the first several days. Subsequently, they recovered until they were identical with the values under normal conditions. The rectal temperature declined during the first load, but after repeated loading decreases of the rectal temperature caused by loading ceased to occur. The changes in the heart rate after administration of methacholine chloride and the results of glucose tolerance tests differed remarkably from the normal during the application of the first immobilization load, but after repeated loading they were the same as in a normal state of rest. In the metabolism of sugar and

lipids in the liver as well, in the seventh load, after repeated loading, there were no longer seen the same pronounced changes that occurred during the first load, and the conditions were the same as in a normal state of rest. It is believed that these facts indicate that the regulatory systems for these functions operate differently from the normal mode of operation during the initial period after the application of immobilization loads. However, as a result of repeated loading, they come to function in a direction where the same homeostasis is maintained as that which applies in the normal state of rest, even during the seventh load. At this point, it is believed that the organism has established adaptation toward immobilization stress.

As the possible mechanism for maintaining the general level of activity of the brain, Magoun et al. [37] suggested a reticular-formation-activating system and Jasper et al. [22] suggested a thalamus-activating system. The fact that the EEG arousal threshold by stimulation of the reticular formation declines as a result of immobilization loads and that the EEG arousal threshold by stimulation of the dorsomedial nucleus of the thalamus increases, indicates that, during immobilization loads, stimuli of the peripheral sensations tend to bring the reticular-formation-activating system into action easily, and that this system plays an important role in the maintenance of the brain's level of activity.

It was found as a result of these experiments, which investigated the electrical activity of the various positions in the brain during the process of application of immobilization stress loads, that the changes in the MUA in the arcuate nucleus of the hypothalamus, the anterior hypothalamic area, and the midbrain reticular formation were constant both during the first immobilization load and during the seventh load. On the other hand, the MUA in the ventromedial nucleus of the hypothalamus, the anterior

hypothalamic area, the preoptic area, and the hippocampus changed during the application of the first load, but there were no changes during the application of the seventh load. These facts indicate that during the process of applying immobilization loads, some of the positions in the brain maintain a fixed reactivity with respect to loads, while others undergo changes in their reactivity, and the brain functions are reorganized. It is believed that the reorganization of the functions of the brain as the center for regulating the functions of the organism is an important process in the establishment of the organism's adaptation to stress.

V. Summary

Mature female rabbits were laid down on their backs, and their four limbs were tied down. These immobilization stress loads were applied repeatedly for 7 days, 6 hours per day, and the changes in the amounts of food and water ingested, the urine volume, and the rectal temperature were investigated. Tests were also made of the changes in the heart rate upon administration of methacholine chloride, the glucose tolerance, and the metabolism of sugar and low-grade fatty acids in the liver slices. Electrodes were also implanted permanently in the brain, the effects of repeated application of immobilization stress loads on the cortical EEG arousal threshold by stimulation of the medial area of the midbrain reticular formation and of the dorsomedial nucleus of the thalamus, and the multiple unit activity (MUA) at various positions in the brain was tested. The following results were obtained.

1. The amounts of food and water ingested and the urine volume declined for the first 2 or 3 days after the commencement of the immobilization loads, but they returned to normal after the 4th day of loading.

2. The rectal temperature declined by 0.6-2.5°C as a result of the application of the first load. However, after repeated

loading, at the time of the seventh load there was no longer any decline of the rectal temperature.

3. During a 1-hour period after intramuscular injection of 0.1 ml of 0.5% methacholine chloride, during the application of the first immobilization load, pronounced differences in the changes undergone by the heart rate were observed as compared with the changes occurring at a state of rest. However, after repeated loading, changes similar to those at a state of rest came to be observed.

4. Glucose tolerance tests were carried out by injecting intravenously 2 ml of 50% glucose. As a result, stagnation of sugar in the blood was observed during the application of the first immobilization load. However, during the application of the seventh immobilization load, the blood sugar was no different from that at a state of rest.

5. C^{14}-u-glucose, as well as C^{14}-labeled acetic acid, propionic acid, and butyric acid were added to liver slices in order to study the metabolism of glucose and low-grade fatty acids. As a result, it was found that during the application of the first immobilization load there were decreases in the glucose consumption and in the inversion to lipids and CO_2, increases in the inversion of low-grade fatty acids to sugar, and decreases in the inversion to lipids and CO_2. However, after repeated applications of immobilization loads, during the seventh load, there ceased to be any effects on 5/122 the metabolism of glucose and low-grade fatty acids.

6. The cortical EEG arousal threshold by stimulation of the midbrain reticular formation decreased as a result of the application of immobilization loads, and the cortical EEG arousal threshold by stimulation of the dorsomedial nucleus of the thalamus increased as a result of the application of immobilization loads.

7. As a result of the application of the first immobilization load, the MUA increased in the ventromedial nucleus of the hypothalamus, the anterior hypothalamic area, and the hippocampus. It decreased in the preoptic area. However, these changes disappeared by the time of the seventh immobilization load. On the other hand, the changes caused by immobilization loads in the MUA of the arcuate nucleus of the hypothalamus, the posterior hypothalamic area, and the midbrain reticular formation were not influenced by repeated applications of immobilization loads. When the first and the seventh loads were applied, there were decreases in the arcuate nucleus of the hypothalamus, increases in the posterior hypothalamic area, increases in the ventromedial region of the midbrain reticular formation, and decreases in the dorsomedial region of the midbrain reticular formation.

On the basis of the above results, it is believed that, after immobilization stress loads had been applied repeatedly for 7 days, the organism came to establish adaptation to immobilization stress and that during the process of repeated application of stress loads the brain functions were reorganized and played an important role in the establishment of adaptation.

In conclusion, the writer wishes to express his deep gratitude to his teacher, Professor Masazumi Kawakami, who kindly provided guidance throughout and who read the manuscript. The writer is also deeply grateful for the kind guidance of Assistant Professor Katsuo Seto. He also wishes to thank Mr. Takashi Higuchi and the other staff members of the Second Physiology Department for their cooperation.

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